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σ^B-Dependent and σ^B-Independent Mechanisms Contribute to Transcription of *Listeria monocytogenes* Cold Stress Genes during Cold Shock and Cold Growth[▽]†

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The role of the stress response regulator σ^B (encoded by *sigB*) in directing the expression of selected putative and confirmed cold response genes was evaluated using *Listeria monocytogenes* 10403S and an isogenic Δ*sigB* mutant, which were either cold shocked at 4°C in brain heart infusion (BHI) broth for up to 30 min or grown at 4°C in BHI for 12 days. Transcript levels of the housekeeping genes *rpoB* and *gap*, the σ^B-dependent genes *opuCA* and *bsh*, and the cold stress genes *ltrC*, *oppA*, and *fri* were measured using quantitative reverse transcriptase PCR. Transcriptional start sites for *ltrC*, *oppA*, and *fri* were determined using rapid amplification of cDNA ends PCR. Centrifugation was found to rapidly induce σ^B-dependent transcription, which necessitated the use of centrifugation-independent protocols to evaluate the contributions of σ^B to transcription during cold shock. Our data confirmed that transcription of the cold stress genes *ltrC* and *fri* is at least partially σ^B dependent and experimentally identified a σ^B-dependent *ltrC* promoter. In addition, our data indicate that (i) while σ^B activity is induced during 30 min of cold shock, this cold shock does not induce the transcription of σ^B-dependent or -independent cold shock genes; (ii) σ^B is not required for *L. monocytogenes* growth at 4°C in BHI; and (iii) transcription of the putative cold stress genes *opuCA*, *fri*, and *oppA* is σ^B independent during growth at 4°C, while both *bsh* and *ltrC* show growth phase and σ^B-dependent transcription during growth at 4°C. We conclude that σ^B-dependent and σ^B-independent mechanisms contribute to the ability of *L. monocytogenes* to survive and grow at low temperatures.

Listeria monocytogenes is a food-borne pathogen that can cause an invasive human illness and that can multiply at temperatures ranging from −0.4 to 45°C (20, 24, 39). Most human listeriosis cases appear to be caused by consumption of refrigerated ready-to-eat foods that permit *L. monocytogenes* growth (37). As the infectious dose for *L. monocytogenes* appears to be high and as initial contamination of processed foods generally occurs at low levels (37), the ability of this organism to grow during refrigerated storage appears to be critical in enabling this pathogen to reach high enough numbers in foods to cause human disease. Consequently, a detailed understanding of the physiology of *L. monocytogenes* during cold shock and cold growth is needed to allow for the design of effective strategies to prevent or minimize *L. monocytogenes* growth on refrigerated ready-to-eat foods and to reduce the number of human listeriosis cases.

Mechanisms that *L. monocytogenes* may use to adapt to low-temperature conditions include the expression of cold shock proteins (CSPs) and cold acclimation proteins (CAPs), changes in membrane lipid composition, and the uptake of osmolytes and oligopeptides (3–5, 8, 10, 29). CSPs and CAPs are defined as proteins showing increased expression during temperature downshift from an organism's optimal growth temperature to a lower temperature; in general, CAPs continue to be expressed at high levels during prolonged cold exposure. In *Escherichia coli* and *Bacillus subtilis*, a number of CSPs have been characterized as RNA chaperones that facilitate the initiation of translation (22, 23). While CSPs and CAPs have also been identified in *L. monocytogenes* (4, 32), the functions of many of these proteins have not yet been clearly defined. One *L. monocytogenes* CSP that is highly synthesized during low-temperature exposure is ferritin (Fri; encoded by *fri*) (21). Fri is involved in hydrogen peroxide resistance and has been hypothesized to be required for iron acquisition (31). However, the specific contributions of Fri to *L. monocytogenes* cold growth and survival are unknown.

In both *L. monocytogenes* and *B. subtilis* exposed to low temperatures, compatible low-molecular-weight solutes can be transported into the bacterial cell and can accumulate to high concentrations (1, 11). In *L. monocytogenes*, carnitine and glycine betaine are the predominant compatible solutes that accumulate during low-temperature exposure and growth (5, 8). These compatible solutes have been shown to stimulate *L. monocytogenes* growth at low temperatures in defined media (1, 2, 5) and function as osmoprotectants (2, 5, 8, 16, 27). Meat and dairy products are rich in carnitine, while glycine betaine is abundant in plants and shellfish (30, 42); therefore, *L. monocytogenes* has access to these compatible solutes in many different foods. The carnitine ATP binding cassette (ABC) transporter system, encoded by the *opuC* operon, and the glycine betaine porter II, encoded by *gbuABC*, are responsible for the uptake of the respective compatible solutes into *L. monocytogenes* (16, 27). In addition to compatible solutes, oligopeptides can be transported into bacterial cells and can accumulate to high concentrations in *L. monocytogenes* exposed to low tem-

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TABLE 1. Primers and TaqMan probes used for this study[a]

Gene	Primers	TaqMan probe[b]
fri	5′-CGGCGGAAGCCCATTC-3′ 5′-CTAAGTCTTCCATTAATTGATCCATTGT-3′	FAM-5′-TATAAGGCGCTTCTTCTACGCTGGCATTTTCT-3′-QSY7
ltrC	5′-CCAGGCATTCTTGCAAAACTAA-3′ 5′-GATGGCGCCGACAATGTC-3′	FAM-5′-CCGTTCACACATTCCTTGA-3′-MGB-NFQ
oppA	5′-GAAGATGCAAAATGGTCAAACG-3′ 5′-TGCACGACGCCATGAGTAAA-3′	FAM-5′-CCTGTAACTGCAAATGACTAT-3′-MGB-NFQ

[a] Primers and TaqMan probes for *gap*, *rpoB*, *opuCA*, and *bsh* have previously been described (33, 34).
[b] QSY7, a nonfluorescent quencher; MGB, minor groove binder; NFQ, 3′ nonfluorescent quencher; FAM, 6-carboxyfluorescein.

peratures (10, 38). Oligopeptide uptake in *L. monocytogenes* is mediated by an oligopeptide permease (encoded by *oppA*) which can transport peptides that are generally ≤8 residues (10, 38). Characterization of an *oppA* null mutant has shown that the peptide transporter encoded by *oppA* is required for *L. monocytogenes* growth at 5°C in brain heart infusion (BHI) medium (10).

Previous studies of the regulation of gene expression during exposure to low temperatures in *L. monocytogenes* (7) and the closely related nonpathogenic bacterium *B. subtilis* (11) have provided preliminary evidence that the stress-responsive alternative sigma factor σ^B (encoded by *sigB*) contributes to the regulation of gene expression in gram-positive bacteria exposed to low temperatures. Specifically, a *B. subtilis sigB* null mutant showed reduced survival at a low temperature (11); in addition, transcription of the *B. subtilis* σ^B regulon appears to be induced at low temperatures (11). In *L. monocytogenes*, σ^B-dependent transcription is induced when the organism enters stationary phase or is subjected to environmental stress conditions, including carbon starvation and acid, osmotic, or oxidative stress (6, 15, 17, 25, 41). Becker et al. (7) reported that σ^B also appears to contribute to the adaptation of stationary-phase *L. monocytogenes* cells to growth at low temperatures. In addition, a number of *L. monocytogenes* genes with confirmed (e.g., *opuCA*, *ltrC*, and *gbu*) or putative (e.g., *fri*) roles in cold growth and adaptation (2, 21, 28, 43, 44) have been shown to be at least partially σ^B dependent (12, 17, 25, 31). We thus hypothesized that σ^B contributes to the regulation of gene expression in *L. monocytogenes* exposed to cold temperatures. To test this hypothesis, we used quantitative reverse transcriptase PCR (qRT-PCR) as well as promoter mapping strategies to characterize the transcription of selected putative and confirmed cold response genes in an *L. monocytogenes* Δ*sigB* null mutant and its isogenic parent strain that had been either (i) cold shocked at 4°C in BHI broth for 30 min or (ii) grown for 12 days at 4°C in BHI.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *L. monocytogenes* serotype 1/2a strain 10403S (9) and an isogenic *sigB* null mutant (Δ*sigB*), FSL A1-254 (41), were used throughout this study. Cells were grown in BHI (Difco, Sparks, MD) at 37°C with shaking (250 rpm). Log-phase cells (optical density at 600 nm [OD_{600}] of 0.4) of both strains were used for all experiments, unless otherwise stated.

Exposure to centrifugation stress. Initial experiments were performed to evaluate the effect of centrifugation on transcript levels of the target genes studied here. Briefly, 5 ml of log-phase *L. monocytogenes* 10403S (OD_{600} = 0.4) were pelleted in a Sorvall RT6000B swing bucket rotor centrifuge (Kendro, Asheville, NC) at 2,190 × *g* for 5 min. Bacterial cells were then resuspended in 250 μl of BHI broth, and cells were added to either (i) 5 ml of prechilled (4°C) BHI or (ii) 5 ml of prewarmed (37°C) BHI broth. Total RNA was isolated from log-phase cells prior to centrifugation as well as from bacterial cells immediately after exposure (i.e., within <30 s) to BHI at 4 or 37°C.

Cold shock and low-temperature growth conditions. To allow for the rapid exposure of *L. monocytogenes* cells to 4°C without prior centrifugation, we initially measured the cooling rate of 8 ml of prewarmed (37°C) BHI broth added to a prechilled (4°C) sterile stainless steel (type 18-8) pan (17.5 cm by 10.8 cm by 5.1 cm; Polarware, Sheboygan, WI). As this protocol allowed for rapid cooling (see Results), it was subsequently used for cold shock experiments that were designed to rapidly expose *L. monocytogenes* cells to 4°C. Briefly, 8 ml of log-phase cultures of *L. monocytogenes* 10403S or the Δ*sigB* strain were cold shocked by adding the culture to a prechilled 4°C pan. A control sample was added to a prewarmed (37°C) stainless steel pan. Bacterial cells were used for RNA isolation as described below either immediately after addition to the covered pan (representing an initial exposure time of <30 s [time zero]) or at 15 or 30 min after addition to the pan. Cells collected from the log-phase culture (37°C) before addition to the stainless steel pans were used as a no-treatment control. Three independent cold shock experiments were carried out for both *L. monocytogenes* 10403S and the Δ*sigB* strain.

For cold growth experiments, log-phase cultures of *L. monocytogenes* 10403S and Δ*sigB* were used to inoculate 10 ml of prechilled BHI (4°C) in 18- by 150-mm disposable culture tubes (borosilicate glass tubes; Fisher Scientific, Pittsburgh, PA) to a starting OD_{600} of 0.15 ± 0.05. Cells were incubated at 4°C without shaking for 12 days, and optical densities were measured every day. Bacterial cells were collected for RNA isolation on days 3, 6, 9, and 12. Three independent replicates of this experiment were performed. In addition, bacterial numbers on days 3, 5, 7, 9, and 11 were determined by spread plating on BHI agar plates.

Total RNA isolation. Total RNA isolation was performed using the RNeasy midi kit (QIAGEN, Valencia, CA) essentially as described by Kazmierczak et al. (26). Briefly, 2 volumes of RNAprotect bacterial reagent (QIAGEN, Valencia, CA) were added to *L. monocytogenes* cells at the time points specified above, followed by cell lysis using QIAGEN′s protocol for "enzymatic lysis with mechanical disruption," except that sonication was performed instead of bead beating as previously described (26). Two DNase treatments (60 min each) were performed on each spin column using 175 μl RNase-free DNase (QIAGEN). After elution from the purification columns, total RNA was precipitated at −80°C and resuspended in RNase-free TE buffer (pH, 8.0; 10 mM Tris and 1 mM EDTA) (Ambion, Austin, TX) immediately before qRT-PCR. Total nucleic acid concentrations (ng/μl) and purity were evaluated using absorbance readings (A_{260} divided by A_{230} and A_{260} divided by A_{280}) determined on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

qRT-PCR. qRT-PCR was performed using a 5′ nuclease (TaqMan) assay format. qRT-PCR primers and TaqMan probes for the housekeeping genes *rpoB* and *gap* as well as for *opuCA* and *bsh* have previously been reported (33, 35). Primer Express 1.0 software (Applied Biosystems, Foster City, CA) was used to design oligonucleotide primers and TaqMan probe sets for *fri*, *oppA*, and *ltrC* (Table 1). TaqMan probes containing the quencher QSY7 or the minor groove binder and a 3′ nonfluorescent quencher were synthesized by Megabases, Inc. (Evanston, IL) or Applied Biosystems.

qRT-PCR was performed using TaqMan one-step RT-PCR master mix reagent (Applied Biosystems) and the ABI Prism 7000 sequence detection system (Applied Biosystems) as described previously by Kazmierczak et al. (26). Control reactions without RT were performed for each template to quantify genomic DNA contamination. As described in detail by Sue et al. (35), standard curves for

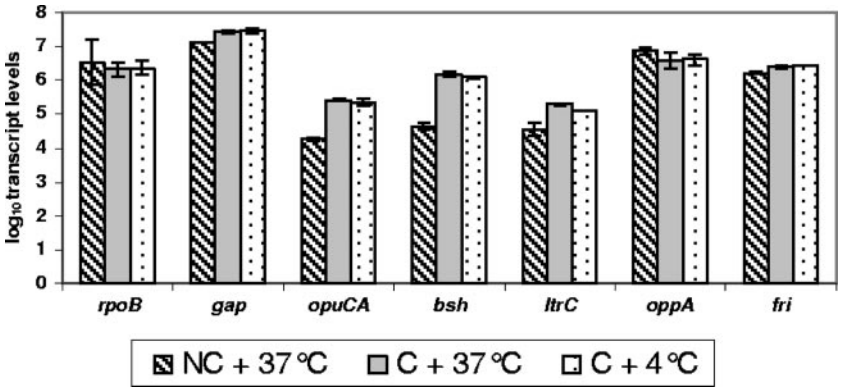

FIG. 1. Log-transformed absolute transcript levels for selected genes before and after centrifugation of *L. monocytogenes*. Transcript levels were determined by qRT-PCR analysis of RNA isolated from (i) log-phase *L. monocytogenes* parent strain cells grown at 37°C prior to centrifugation, (ii) log-phase *L. monocytogenes* parent strain cells centrifuged and immediately exposed for <30 s to fresh BHI prewarmed to 37°C, and (iii) log-phase *L. monocytogenes* parent strain cells centrifuged and immediately exposed to fresh BHI prechilled to 4°C for <30 s. Values shown represent the averages of results from qRT-PCR assays performed on two independent RNA collections. Error bars indicate the data range. NC, no centrifugation; C, centrifugation.

each target gene were included for each assay to allow absolute quantification of cDNA levels. Data were analyzed using the ABI Prism 7000 sequence detection system software (Applied Biosystems) as previously described (35).

RACE-PCR. A 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen, Carlsbad, CA) was used as previously described (25) to map the transcriptional start sites for selected genes. Briefly, mRNA was reverse transcribed using a gene-specific primer (GSP1) and tailed with dCTP using terminal deoxynucleotidyl transferase. Poly(dC)-tailed cDNA was amplified using a nested gene-specific primer (GSP2) and a poly(G/I) primer (provided in the RACE-PCR kit) using AmpliTaq Gold (Applied Biosystems) and a touchdown PCR protocol. Gene-specific primers included *ltrC* GSP1 (5′-AACTGCGTGCTTTTGATTCT-3′), *ltrC* GSP2 (5′-AGGCACTTTGTTTTTCTACCA-3′), *oppA* GSP1 (5′-AAT TAGTGCGCTTTCTGTCAA-3′), *oppA* GSP2 (5′-CCTCCGCATGCTACCAA GAC-3′), *opuCA* PsigA GSP1 (5′-ATCGATAACTAATTTCCCTAACTA-3′), and *opuCA* PsigA GSP2 (5′-CAGTGTTACATTCAAACGGAAGT-3′). In addition, previously described gene-specific primers for *opuCA* were used (25). PCR products were separated on 3% agarose gels; DNA fragments of interest were purified using a QIAquick gel extraction kit (QIAGEN) and cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen). Plasmids containing an insert were sequenced at the Cornell University Bioresource Center (Ithaca, NY). Sequences were analyzed using Lasergene v6.0 (DNAstar, Inc., Madison, WI).

Statistical analyses. An analysis of variance (ANOVA) to determine if the OD_{600} values of *L. monocytogenes* 10403S and the Δ*sigB* strain grown at 4°C were affected by strain (parent strain or Δ*sigB* strain), replicate, or time of incubation was performed with the Proc general linear model (GLM) function of SAS (version 8e; SAS Institute, Cary, NC) using a split-plot model. Bacterial strain and replicate were designated category variables. Day of incubation was treated as a continuous variable in the ANOVA. Distortion of the ANOVA by multicollinearity of the linear and quadratic terms for time of incubation was minimized by centering the incubation times using a mathematical transformation (19). The day of incubation time was transformed as follows: day transformed = day of storage − [(last storage day − first storage day)/2]. This transformation made the data set orthogonal with respect to time. The whole plot error term (strain by replicate interaction) was used as the error term to test the significance of strain and replicate. The model was considered significant if the F test had a P of <0.05.

Transcript levels for target genes determined by qRT-PCR data were $\log_{10}$ transformed and then normalized to the geometric mean of *rpoB* and *gap* transcript levels as previously described (26); $\log_{10}$ ratios less than 0 thus indicate that the target gene had lower transcript levels than the average transcript levels for *rpoB* and *gap*, which are highly transcribed. Statistical analysis of qRT-PCR data was performed as described by Kazmierczak et al. (26) using a GLM with multiple comparisons performed using Tukey's studentized range test (implemented as least-square [LS] means in SAS). Cell collection date, RNA collection date, and qRT-PCR assay date were used as blocking factors in a mixed-effects model for selected genes if these factors were found to be significant in the initial GLM analysis. A P value of <0.05 was considered significant.

RESULTS

Centrifugation increases transcript levels for σ^B-dependent genes, requiring the development of a centrifugation-independent protocol for cold shock exposure. In initial experiments, we found that the transcript levels of the σ^B-dependent genes *opuCA*, *bsh*, and *ltrC* were consistently higher for cells collected immediately after a centrifugation step (regardless of whether the cells were resuspended in BHI tempered at 4 or 37°C) than for cells collected for RNA isolation immediately before RNA stabilization (Fig. 1). On the other hand, centrifugation did not show any apparent effect on transcript levels for *fri* and genes that are not σ^B dependent (*rpoB*, *oppA*, and *gap*). As these preliminary results were consistent with observations by Chaturongakul and Boor (13), who also found increased transcript levels for σ^B-dependent genes after centrifugation, we devised a cold shock exposure protocol for *L. monocytogenes* that did not include a centrifugation step. Specifically, 8 ml of log-phase *L. monocytogenes* cells grown in BHI at 37°C was pipetted into prechilled (4°C) sterile stainless steel pans, and the temperature of the broth was measured at 1-min intervals for 15 min. The temperature of the broth reached 4°C after 6 min (see Fig. S1 in the supplemental material), indicating that this protocol was suitable for creating cold shock exposure in *L. monocytogenes* cells that had been grown at 37°C, without a centrifugation step.

Cold shock exposure to 4°C for 30 min does not induce the transcription of *ltrC*, *oppA*, *opuCA*, or *fri* but induces the σ^B-dependent transcription of *bsh*. To determine if σ^B-dependent transcription is activated during cold shock, log-phase cells of *L. monocytogenes* 10403S and the Δ*sigB* strain that had been grown at 37°C were cold shocked by rapidly cooling them in prechilled (4°C) sterile stainless steel pans with cell collection for qRT-PCR at the initial time point (0 min, i.e., <30 s after addition to the pan) and after 15 and 30 min. As a control, an equal aliquot of the same culture was also transferred to prewarmed (37°C) pans. Bacterial cells were also collected for qRT-PCR from the culture immediately before cells were added to the pans. Transcript levels did not differ significantly

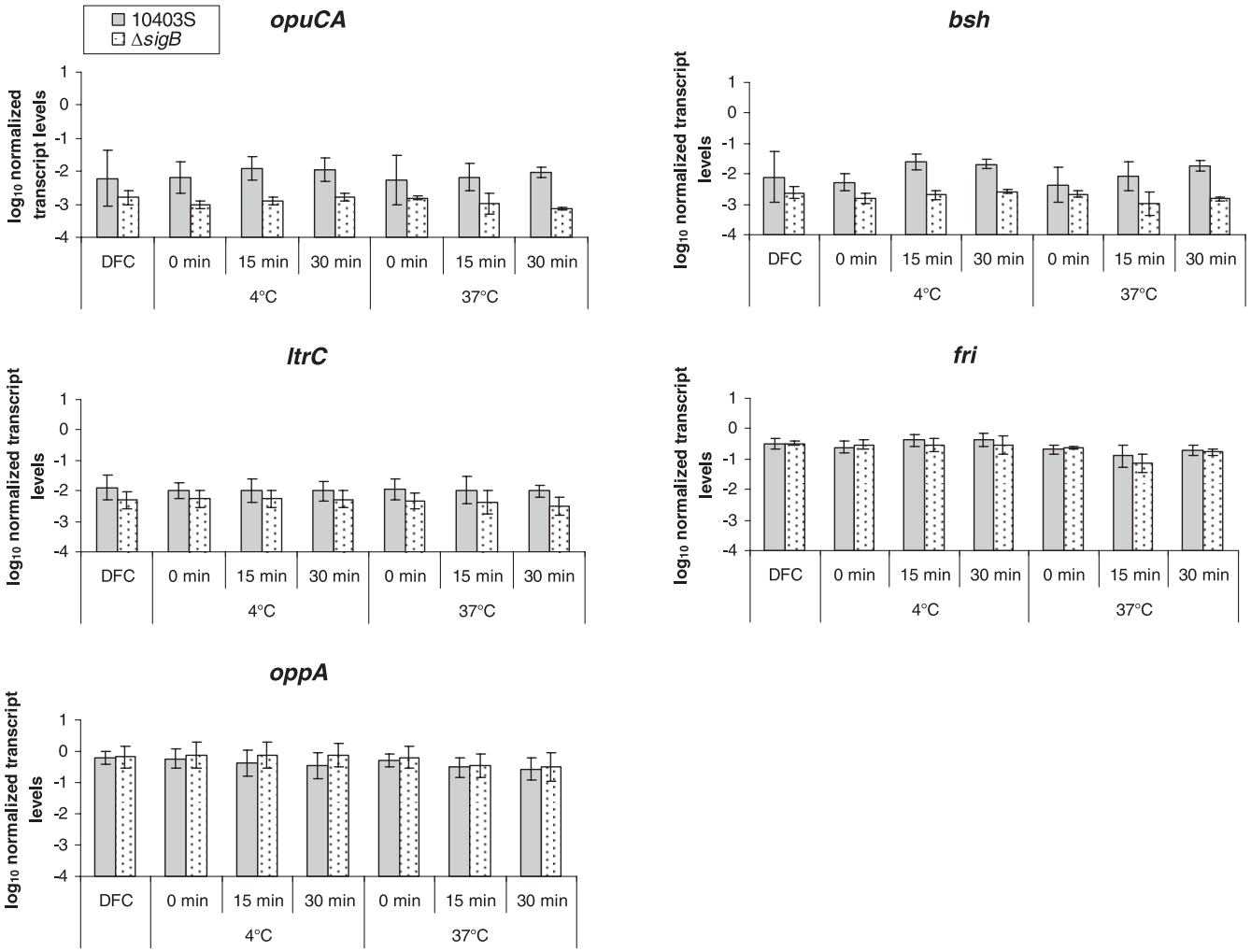

FIG. 2. Normalized, log-transformed transcript levels for *opuCA*, *bsh*, *ltrC*, *oppA*, and *fri* during the cold shock at 4°C and during control exposure to 37°C of the *L. monocytogenes* parent strain and a Δ*sigB* mutant. Transcript levels were determined by qRT-PCR analysis of RNA isolated from the (i) log-phase *L. monocytogenes* parent strain (10403S) and Δ*sigB* strain before exposure (directly from the culture [DFC]); (ii) parent strain and Δ*sigB* strain cold shocked at 4°C in prechilled stainless steel pans for <30 s (0 min), 15 min, and 30 min; and (iii) parent strain and Δ*sigB* mutant exposed to 37°C in prewarmed stainless steel pans for <30 s (0 min), 15 min, and 30 min. Transcript levels for each gene were log transformed and normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Values shown represent the averages of results from qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations. Results of statistical analyses using ANOVA are detailed in the text and shown in Table S1 in the supplemental material.

(P was >0.05 as determined by ANOVA using data for all genes before exposure and after exposure for 0 min to 37°C) between cells before exposure and immediately after exposure to 37°C (Fig. 2).

Overall, *opuCA* and *bsh* transcript levels were consistently higher in *L. monocytogenes* 10403S than in the Δ*sigB* mutant (Fig. 2), and the transcript levels for these two genes were significantly affected by the factor "strain" ($P < 0.0001$, GLM analysis), consistent with previous observations that these two genes are σ^B dependent (34). In addition, *ltrC* transcript levels were also consistently higher in 10403S than in the Δ*sigB* mutant (Fig. 2) and were significantly affected by the factor "strain" ($P < 0.05$, GLM analysis), indicating that *ltrC* transcription is also σ^B dependent. Interestingly, *bsh* transcript levels increased over time in parent strain cells exposed to 4°C, with transcript levels at both 15 and 30 min after cold exposure significantly (P was <0.05 as determined by LS means analysis of 4°C data for the parent strain) higher than transcript levels at 0 min at 4°C, indicating induction of this σ^B-dependent gene during cold shock at 4°C. The factor "time" significantly affected *bsh* transcript levels in 10403S at 4°C, while there was no significant effect of time on *bsh* transcript levels in the Δ*sigB* mutant at 4°C or on *bsh* levels in either the Δ*sigB* mutant or the parent at 37°C (see Table S1 in the supplemental material). Combined, these results indicate that *bsh* induction at 4°C is σ^B dependent (as no induction was observed in the Δ*sigB* mutant) and occurs during cold shock but not at 37°C.

The factor "temperature" had a significant effect only on *fri* and *oppA* transcript levels ($P < 0.0001$, mixed-effects analysis). The mean-normalized, log-transformed transcript levels for *fri* and *oppA* at 4°C (−0.49 and −0.24, respectively [averages of values at all three time points, i.e., 0, 15, and 30 min]) were

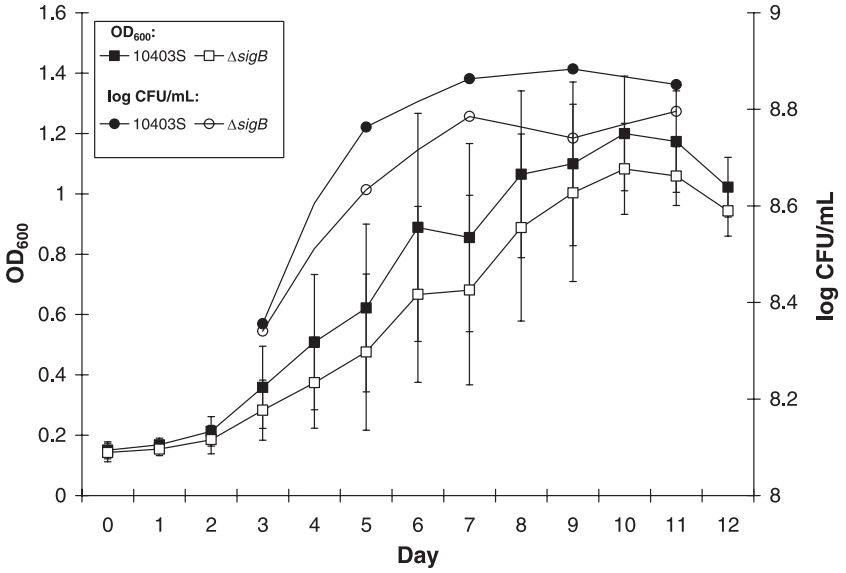

FIG. 3. Growth of the *L. monocytogenes* parent strain 10403S and the Δ*sigB* strain at 4°C. The OD_{600}s of the *L. monocytogenes* parent strain 10403S and Δ*sigB* mutant were measured during growth at 4°C for 12 days. Data on the left *y* axis show the average OD_{600} values from five independent experiments; error bars indicate standard deviations. Data on the right *y* axis show the average cell counts for *L. monocytogenes* strain 10403S and the Δ*sigB* mutant determined in one experiment.

higher than the mean transcript levels for these genes at 37°C (−0.79 and −0.42, respectively, for *fri* and *oppA* [averages of results at all three time points]). Transcript levels for cells prior to exposure (−0.49 and −0.19 for *fri* and *oppA*, respectively) were similar to those for cells exposed to 4°C but higher than those for cells exposed to 37°C. Thus, rather than being upregulated during exposure to 4°C, transcript levels for these two genes appear to be lower in cells exposed to 37°C.

Expression of the putative cold stress genes *opuCA*, *fri*, and *oppA* is predominantly σ^B independent during growth at 4°C, as shown by mRNA transcript levels. While OD_{600} measurements and cell enumeration showed that *L. monocytogenes* 10403S and the Δ*sigB* strain have similar growth patterns over 12 days in BHI at 4°C (Fig. 3), the OD_{600} values for the Δ*sigB* strain were consistently slightly lower than the OD_{600} values for the isogenic parent strain (10403S). While statistical analysis confirmed that there was a significant effect (P was 0.01 as determined by GLM analysis using a split-plot model) of the factor "strain" (i.e., the parent strain or Δ*sigB* strain) on OD_{600} measurements of *L. monocytogenes* cells grown at 4°C, statistical analyses of cell enumeration data performed in triplicate indicated no significant effect of "strain" on *L. monocytogenes* 10403S and Δ*sigB* strain numbers for bacteria grown at 4°C for 12 days (Y. C. Chan, Y. Hu, S. Chaturongakul, K. D. Files, B. Bowen, K. J. Boor, and M. Wiedmann, submitted for publication). Thus, the parent and the Δ*sigB* strain do not appear to differ in their abilities to grow in BHI at 4°C.

In order to determine the contributions of σ^B to the transcription of *L. monocytogenes* genes during growth at 4°C, transcript levels for selected *L. monocytogenes* genes were determined using qRT-PCR with 10403S and the Δ*sigB* mutant grown at 4°C for 3, 6, 9, and 12 days (Fig. 4). Analysis of normalized transcript levels showed that *bsh* and *ltrC* transcript levels were consistently lower in the Δ*sigB* mutant than in the parent strain (Fig. 4); statistical analysis using GLM also showed that the factor "strain" (i.e., 10403S or the Δ*sigB* mutant) has a significant effect on *bsh* ($P < 0.0001$) and *ltrC* ($P < 0.005$) transcript levels (see Table S2 in the supplemental material). *opuCA*, *fri*, and *oppA* transcript levels were similar in the parent strain and the Δ*sigB* mutant (Fig. 4) and were not affected by the factor "strain" ($P > 0.05$, GLM analysis). For *opuCA*, this finding was initially surprising as this gene has been identified as σ^B dependent in multiple studies (12, 13, 16, 17, 25, 26, 34, 35). The results were subsequently explained by identification of a σ^B-independent promoter that is active at 4°C and located upstream of *opuCA* (see below).

Transcript levels for the σ^B-dependent genes *bsh* and *ltrC* were significantly affected by the factor "time" (P was <0.001 as determined by GLM analysis using data for the parent and the Δ*sigB* strain [see Table S2 in the supplemental material]), although *opuCA*, *fri*, and *oppA* transcript levels were not. *bsh* transcript levels in the parent strain grown for 6, 9, and 12 days at 4°C were significantly higher ($P < 0.05$, LS means analysis) than those of the parent strain grown for only 3 days at 4°C. *ltrC* transcript levels in the parent strain showed similar trends, even though only *ltrC* transcript levels at days 9 and 12 were significantly higher ($P < 0.05$, LS means analysis) than those at day 3. These findings for these two σ^B-dependent genes are consistent with observations that σ^B is a stationary-phase alternative sigma factor (6, 34) and indicate that both σ^B activity and σ^B-dependent transcription increase as *L. monocytogenes* cells enter stationary phase at 4°C.

Initial analysis of absolute transcript levels for *rpoB* and *gap* in *L. monocytogenes* cells grown at 4°C showed that transcript levels for *rpoB* were significantly affected by the factors "time" and "strain" (with transcript levels at days 9 and 12 being lower than transcript levels at days 3 and 6), while transcript levels for *gap* were not significantly affected by these two factors.

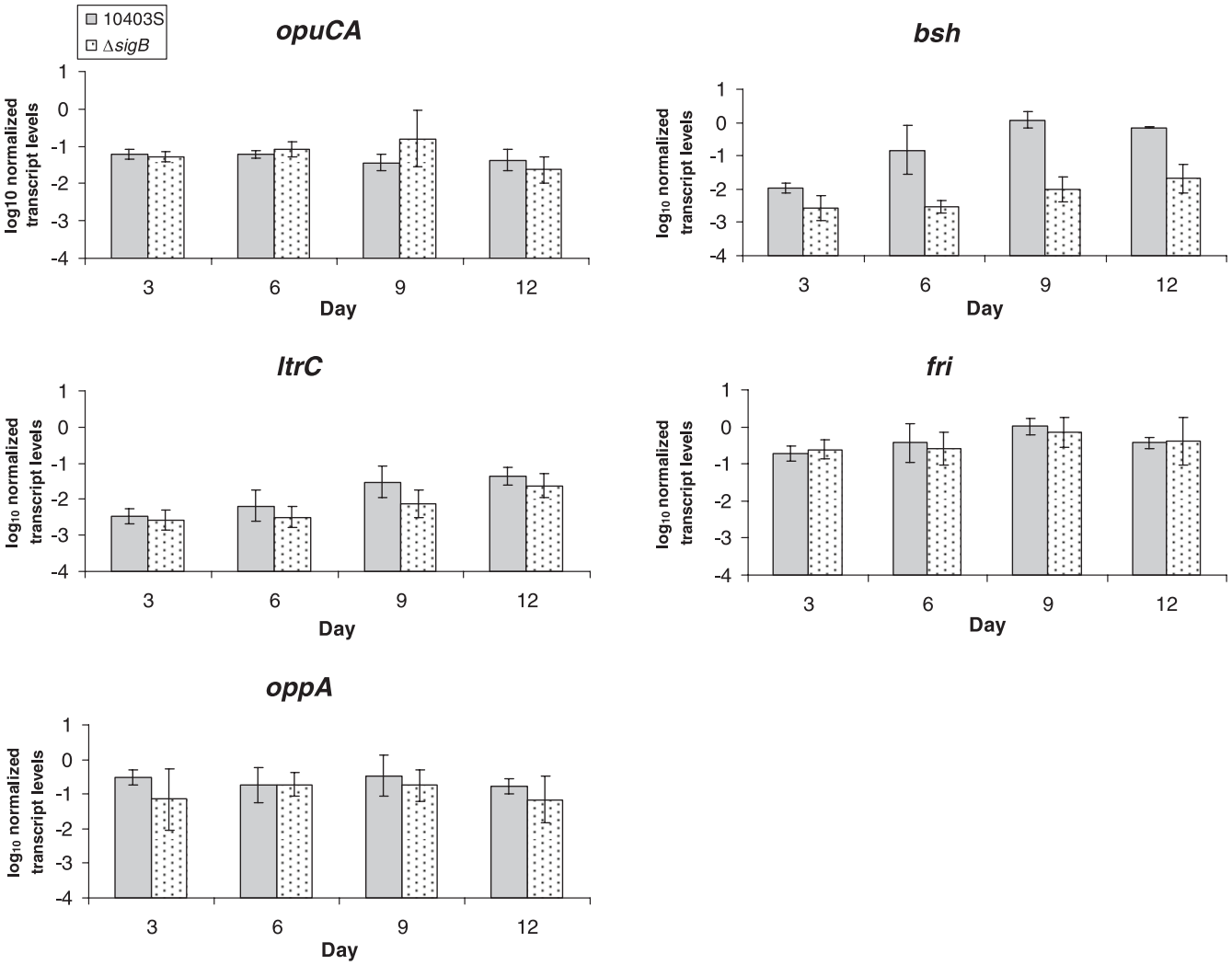

FIG. 4. Normalized, log-transformed transcript levels of *rpoB*, *gap*, *opuCA*, *bsh*, *ltrC*, *oppA*, and *fri* for the *L. monocytogenes* parent strain and the Δ*sigB* strain grown at 4°C. Transcript levels were determined by qRT-PCR analysis of RNA isolated from the *L. monocytogenes* parent strain 10403S and the Δ*sigB* strain grown in BHI at 4°C for 3, 6, 9, and 12 days. Transcript levels for each gene were log transformed and normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Values shown represent the averages of results of qRT-PCR assays performed on three independent RNA collections; error bars show standard deviations. Results of statistical analyses using ANOVA are detailed in the text and shown in Table S2 in the supplemental material.

While we still elected to normalize transcript levels for all target genes (i.e., *oppA*, *ltrC*, *bsh*, *fri*, and *opuCA*) to the geometric means of the *rpoB* and *gap* transcript levels, providing (i) for more robust normalization due to the use of two rather than one housekeeping gene and (ii) for comparisons with other data that used the same two housekeeping genes for normalization (e.g., see reference 26), we also reanalyzed our data using *ltrC* and *bsh* transcript levels normalized to *gap* only (as *ltrC* and *bsh* were the only two genes that showed significant differences in transcript levels). The results from both analyses were consistent; in both analyses, *ltrC* and *bsh* transcript levels were significantly ($P < 0.05$) affected by the factors "time" and "strain."

***ltr*C and *fri* are transcribed from at least one σ^B-dependent promoter as revealed by RACE-PCR.** To further test whether transcription of the cold stress genes *ltrC*, *fri*, and *oppA* is σ^B dependent, a RACE-PCR protocol was used to map transcriptional start sites and identify the promoters for these genes. For *ltrC*, we found a single σ^B-specific transcript (i.e., a transcript was amplified in 10403S but was absent in the Δ*sigB* strain) (Fig. 5A). Sequencing of this σ^B-specific *ltrC* RACE-PCR product allowed us to map the transcriptional start site for this transcript and identified a putative σ^B-dependent promoter (Fig. 5B) that closely matches the σ^B consensus promoter described by Kazmierczak et al. (25) but that differs from the putative σ^B-dependent *ltrC* promoter previously identified by visual inspection of the DNA sequence upstream of this gene (25). The −10 region of this promoter is located approximately 40 nucleotides upstream of the *ltrC* start codon (Fig. 5B). For *fri*, RACE-PCR identified a σ^B-independent transcript (i.e., a transcript was amplified in both 10403S and the Δ*sigB* strain) (Fig. 5A) as well as a σ^B-specific transcript (i.e., a transcript was amplified in 10403S but was absent from the Δ*sigB* strain) (Fig. 5A). Despite repeated attempts, we

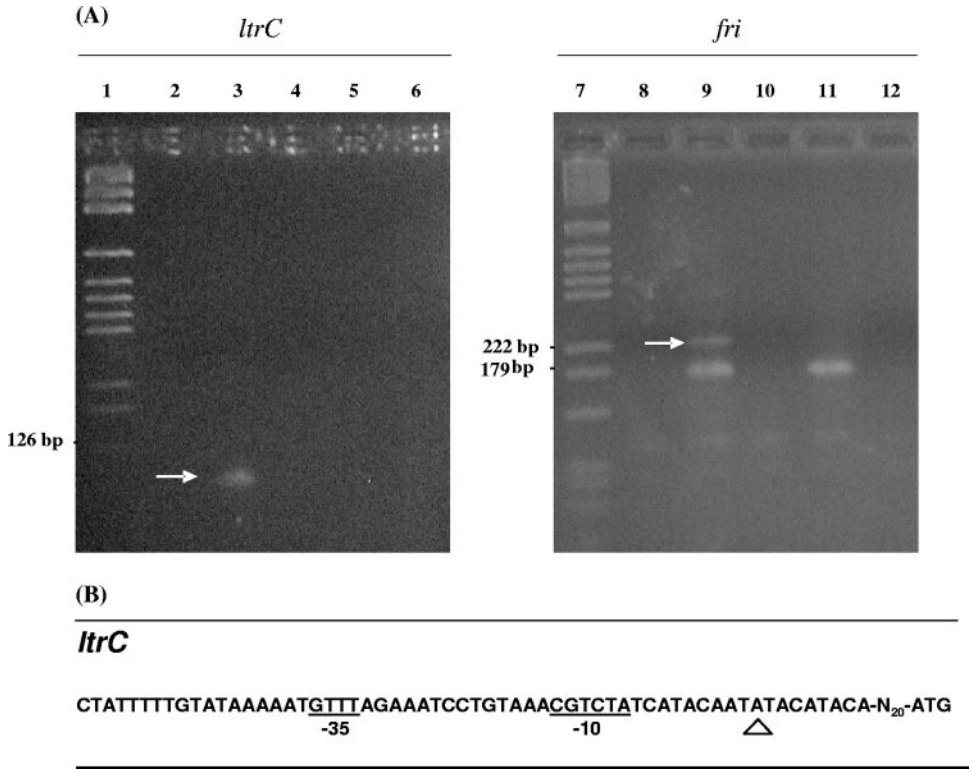

FIG. 5. RACE-PCR and promoter sequences of *ltrC* and *fri*. RACE-PCR was performed using total RNA collected from *L. monocytogenes* parent strain (10403S) and Δ*sigB* cells cold shocked at 4°C for 30 min. (A) Agarose gel electrophoresis of RACE-PCR products for *ltrC* (lanes 2 to 6) and *fri* (lanes 8 to 12). Lanes 1 and 7, pGEM DNA size marker (Promega, Madison, WI); lanes 2 and 8, PCR of untailed *L. monocytogenes* parent strain cDNA (negative control); lanes 3 and 9, PCR of poly(dC)-tailed parent strain cDNA; lanes 4 and 10, PCR of untailed *L. monocytogenes* Δ*sigB* cDNA (negative control); lanes 5 and 11, PCR of poly(dC)-tailed Δ*sigB* cDNA; lanes 6 and 12, negative-control PCR with no cDNA. Arrows indicate σ^B-dependent transcripts (i.e., transcripts that were detected in RNA from the parent strain but not in RNA from the Δ*sigB* strain). The contrast of the *ltrC* gel picture was enhanced to visualize DNA fragments. (B) σ^B promoter sequence for *ltrC* determined by RACE-PCR. The −35 and −10 regions are underlined. The triangle indicates the transcriptional start site determined by RACE-PCR.

were unable to clone the σ^B-specific RACE-PCR product for *fri*, most likely due to the presence of a larger quantity of a σ^B-independent RACE-PCR product of similar size. However, Olsen et al. (31) recently used primer extension to identify a putative σ^B-dependent promoter upstream of *fri*, which is consistent with the size of the RACE-PCR product observed here. For *oppA*, RACE-PCR identified only a σ^B-independent transcript (i.e., a transcript that was amplified in both 10403S and the Δ*sigB* strain).

***opuCA* is transcribed from a σ^A-dependent promoter during growth at a low temperature and in the absence of σ^B expression.** Our data indicated that *opuCA* transcription in *L. monocytogenes* grown at 4°C was σ^B independent, while *opuCA* transcription during cold shock (our data reported here) and during growth at 30 and 37°C (25, 34) was σ^B dependent, so we used RACE-PCR to map *opuCA* transcriptional start sites in 10403S and Δ*sigB* cells grown to stationary phase (day 9) at 4°C or grown to stationary phase (OD_{600} = 2.0) at 37°C. For *L. monocytogenes* 10403S and the Δ*sigB* strain grown at 4°C, RACE-PCR identified a single σ^B-independent transcript (i.e., transcripts were amplified in both 10403S and the Δ*sigB* strain) but no σ^B-dependent transcript, indicating σ^B-independent transcription of *opuCA* at 4°C. Sequencing of this RACE-PCR product identified a putative σ^A-dependent promoter upstream of this transcriptional start site (Fig. 6B). For stationary-phase *L. monocytogenes* grown at 37°C, RACE-PCR identified a σ^B-specific transcript (i.e., the transcript was amplified in 10403S but was absent from the Δ*sigB* strain) (Fig. 6A) as well as a σ^B-independent transcript (found only in the Δ*sigB* strain), which matched the size of the σ^B-independent transcript amplified in *L. monocytogenes* grown at 4°C. Sequencing of these PCR products identified transcriptional start sites that corresponded to the putative σ^A-dependent promoter identified in cells grown at 4°C as well as to the σ^B-dependent promoter previously identified upstream of *opuCA* (25). As the σ^B-independent RACE-PCR product was not detected in *L. monocytogenes* 10403S grown at 37°C (possibly due to the abundance of the σ^B-dependent transcript in this strain, which might be amplified preferentially during cDNA synthesis relative to the larger σ^A-dependent transcript), we designed gene-specific RACE-PCR primers (*opuCA* PsigA GSP1 and GSP2) to exclusively reverse transcribe and amplify the σ^A-dependent *opuCA* transcript. RACE-PCR with these primers yielded a σ^B-independent RACE-PCR product (which mapped to the same σ^A-dependent promoter identified above) in both *L. monocytogenes* 10403S and the Δ*sigB* strain cells grown to stationary phase at 37°C. Thus, the σ^A-dependent promoter iden-

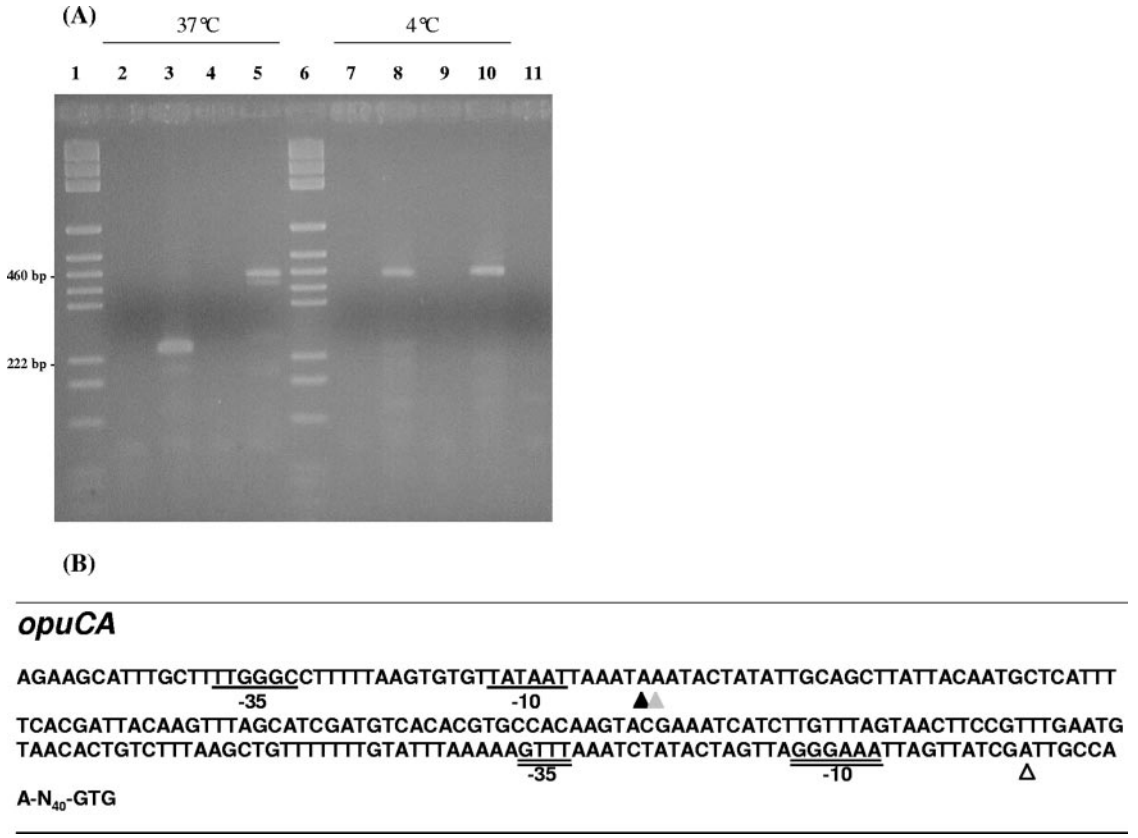

FIG. 6. RACE-PCR and promoter sequence of *opuCA*. RACE-PCR was performed using total RNA collected from the *L. monocytogenes* parent strain and Δ*sigB* strain after growth to stationary phase (day 9) at 4°C or after growth to stationary phase (OD_{600} = 2.0) at 37°C and previously described *opuCA*-specific primers (25). (A) Agarose gel electrophoresis of RACE-PCR products for *opuCA* in *L. monocytogenes* grown to stationary phase in BHI at 37°C (lanes 2 to 5) or at 4°C (lanes 7 to 10). Lanes 1 and 6, pGEM DNA size marker; lanes 2 and 7, PCR of untailed *L. monocytogenes* parent strain (10403S) cDNA (negative control); lanes 3 and 8, PCR of poly(dC)-tailed parent strain cDNA; lanes 4 and 9, PCR of untailed Δ*sigB* strain cDNA (negative control); lanes 5 and 10, PCR of poly(dC)-tailed Δ*sigB* cDNA; lane 11, negative-control PCR with no cDNA. (B) σ^B and σ^A promoter sequences for *opuCA* as determined by RACE-PCR. The −35 and −10 regions for the σ^B- and σ^A-dependent promoters are double and single underlined, respectively. Triangles indicate transcriptional start sites for *opuCA* determined by RACE-PCR in the *L. monocytogenes* parent strain (△) and Δ*sigB* mutant (△) grown at 37°C and for the parent strain and Δ*sigB* mutant grown at 4°C (▲).

tified upstream of *opuCA* also contributes to *opuCA* transcription at 37°C in a *L. monocytogenes* strain with a functional σ^B.

DISCUSSION

To determine the role of σ^B during cold shock and growth at a low temperature, *L. monocytogenes* 10403S and an isogenic Δ*sigB* null mutant were (i) exposed to cold shock conditions or (ii) grown at 4°C over 12 days. qRT-PCR using mRNA isolated from these cells was used to measure transcript levels for selected cold stress genes and σ^B-dependent genes and to map, using RACE-PCR, transcriptional start sites of selected genes. We confirmed that centrifugation rapidly induces σ^B-dependent transcription, which necessitated the use of centrifugation-independent protocols to evaluate the contributions of σ^B to transcription during cold shock. Our data indicate that (i) σ^B is not required for *L. monocytogenes* growth at 4°C in BHI; (ii) transcription of the cold stress genes *ltrC* and *fri* is at least partially σ^B dependent; (iii) while σ^B activity is induced during 30 min of cold shock at 4°C, this cold shock does not induce the transcription of σ^B-dependent or -independent cold shock genes; and (iv) expression of the putative cold stress genes *opuCA*, *fri*, and *oppA* is σ^B independent during growth at 4°C, while the cold shock gene *ltrC* as well as *bsh* shows growth phase and σ^B-dependent transcription during growth at 4°C.

Centrifugation rapidly induces σ^B-dependent transcription. Consistently with a previous report (13), we found that transcript levels of the σ^B-dependent genes *opuCA*, *bsh*, and *ltrC* increased following centrifugation. Centrifugation prior to RNA stabilization should thus be avoided in experiments aimed at evaluating the effects of σ^B on gene transcription. The inclusion of a centrifugation step may result in underestimation of σ^B's contributions to gene transcript levels or phenotypic measurements if both control and experimental treatments are exposed to centrifugation, as σ^B-dependent transcription would also be induced in the control. On the other hand, experiments where only the treatment group is exposed to centrifugation may overestimate the contribution of σ^B, as centrifugation rather than the intended treatment could induce σ^B activity. Consequently, the cold shock procedures used in the study reported here differed from those used in previous studies that included a centrifugation step (29, 36, 40). As previous studies have shown that activation of σ^B under

stress conditions occurs rapidly (i.e., <5 min [35]), we also chose to avoid cold shock protocols in which bacterial cultures are cooled in glass flasks, as these protocols produce a slow "temperature downshift" from 37°C to 4°C, which can take 30 min or more (14, 18). To allow for rapid cold shock exposure of *L. monocytogenes* without centrifugation, we thus developed a cold shock protocol that entailed rapid cooling of bacterial cultures in sterile, large-surface stainless steel pans.

σ^B is not required for *L. monocytogenes* growth at 4°C. While our results indicate that an *L. monocytogenes* strain with a nonpolar Δ*sigB* deletion did not show reduced growth at 4°C in BHI compared to that of its isogenic parent strain, despite lower OD_{600} values for the Δ*sigB* strain, others (7) have reported the reduced growth at 4°C of *L. monocytogenes* 10403S with an insertional mutation in *sigB* in defined medium, which is less nutrient rich than BHI. These discrepancies may result from σ^B-independent effects of the insertional mutagenesis approach used by Becker et al. (7), e.g., growth retardation at 4°C due to the presence and expression of an antibiotic resistance gene in the insert. Alternatively, a *sigB* deletion may affect *L. monocytogenes* growth only at low temperatures in minimal media, e.g., as growth in these media may also represent a stress condition unrelated to temperature. Further characterization of *L. monocytogenes* strains with in-frame *sigB* deletions for the ability to grow in different media and in selected foods at 4 and 30 or 37°C will be necessary to further dissect possible contributions of σ^B to cold growth under different environmental conditions.

Transcription of the cold stress genes *ltrC* and *fri* is at least partially σ^B dependent. The transcription of *opuCA* and the *opuCABCD* operon, which encodes a carnitine transporter that appears to be involved in enhancing *L. monocytogenes* survival under conditions of osmotic and cold stress (2), has been found to be at least partially σ^B dependent in a number of studies (12, 16, 17, 25, 34). Our study confirmed that other genes with putative or confirmed roles in cold stress survival/cold growth (i.e., *ltrC* and *fri*) are also at least partially σ^B dependent. *ltrC*, which encodes a protein termed low-temperature requirement C protein, with an as-yet-unknown function, has previously been shown to be required for the growth of *L. monocytogenes* at 4°C in BHI (43, 44) with antibiotics, which were needed to maintain the transposon insertion in *ltrC* in the strain used. Our results are consistent with a previous microarray study which showed that *ltrC* transcript levels are σ^B dependent in *L. monocytogenes* exposed to osmotic-stress conditions (25) and which found that *ltrC* is preceded by a putative σ^B-dependent promoter. We experimentally identified a σ^B-dependent *ltrC* promoter and thus showed that *ltrC* transcription can directly be activated by σ^B. Our data on the transcription of *fri*, which encodes an 18-kDa CSP hypothesized to be required for iron storage in *L. monocytogenes* (31), are consistent with a study by Olsen et al. (31) which mapped the σ^B- and σ^A-dependent promoter regions of *fri* and found that *fri* was partially σ^B dependent at 37°C. Importantly, our data show that *fri* is transcribed using both σ^B-dependent and -independent mechanisms in *L. monocytogenes* exposed to 4°C. Our data also show that the transcription of *oppA*, which encodes an oligopeptide permease that is required during growth at low temperatures (10), is clearly σ^B independent. Overall, we conclude that, among selected genes that have previously been reported to contribute to cold growth or to be induced by low temperatures, some genes are partially σ^B dependent while others are clearly σ^B independent. Even those genes with contributions to cold growth that are preceded by a σ^B-dependent promoter and clearly show σ^B-dependent transcription under certain conditions may be predominantly expressed through σ^B-independent mechanisms at 4°C, as was strikingly demonstrated by the σ^B-independent transcription of *opuCA* during growth at 4°C.

While σ^B activity is induced during 30 min of cold shock at 4°C, this cold shock does not lead to the induction of σ^B-dependent or -independent cold shock genes. Measurements of transcript levels of selected genes in the *L. monocytogenes* 10403S and Δ*sigB* strains during 4°C cold shock for 30 min revealed that σ^B activity is induced within 15 min of exposure of *L. monocytogenes* to 4°C, as supported by increased transcription of the σ^B-dependent *bsh* gene in 10403S. While *bsh* does not have a recognized role during growth at low temperatures, it appears to be a suitable indicator gene for σ^B activity under these conditions. *opuCA* has been used as an indicator gene for σ^B activity in a number of previous studies (35); however, our results show σ^B-independent *opuCA* transcription, particularly at 4°C. The rapid induction of σ^B activity reported here is consistent with previous studies that have shown the induction of σ^B activity within <5 min of *L. monocytogenes* exposure to other stress conditions (e.g., osmotic stress) (35).

Interestingly, in our study, neither the σ^B-independent gene *oppA* nor the at least partially σ^B-dependent cold shock genes *fri*, *opuCA*, and *ltrC* showed induction of transcription during cold shock exposure for 30 min. In contrast, Graumann et al. (18) showed that the production of 23 proteins was induced within 30 min of cold shock (i.e., exposure to 15°C) in *B. subtilis*. While Angelidis et al. (2) reported increased carnitine uptake through activation of a transporter encoded by *opuC* in *L. monocytogenes* exposed to cold shock for 30 min in defined media with carnitine, their protocol used a centrifugation step. In addition, increased carnitine uptake could be mediated by protein activation or increased translation of the preformed *opuC* transcript rather than induction of transcription. While our findings may indicate that rapid induction of cold shock genes is limited in the transition of log-phase *L. monocytogenes* cells from higher temperatures (e.g., 37°C) to low temperatures in BHI, future full-genome transcriptional profiling during cold shock is needed to determine whether there are any specific *L. monocytogenes* genes that are rapidly induced during cold shock. The identification of σ^B-independent promoters upstream of *fri* and *opuCA* provides a possible explanation for the observation that transcription of these genes is not induced during a 30-min cold shock, even though σ^B activity seems to be induced; enhanced σ^B-dependent transcription of these genes may be too limited to be detected above baseline levels of σ^B-independent transcription. Alternatively or in addition, only part of the σ^B regulon may be induced during cold shock and the induction of σ^B-dependent transcription under cold shock conditions may require transcriptional activators in addition to σ^B. This hypothesis is consistent with microarray-based data that indicated that different members of the σ^B regulon are induced under different σ^B-activating stress conditions (i.e., osmotic or stationary-phase stress) (25).

Expression of the putative cold stress genes *opuCA*, *fri*, and *oppA* is σ^B independent during growth at 4°C, while the cold shock gene *ltrC* as well as *bsh* shows growth phase and σ^B-dependent transcription during growth at 4°C. Consistently with the fact that σ^B is a stationary-phase σ factor and that σ^B activity is induced in *L. monocytogenes* cells grown to stationary phase at 30 and 37°C (25, 34), we found that the transcription of *bsh* and *ltrC*, both of which are preceded by σ^B-dependent promoters, is higher in stationary-phase cells grown at 4°C than in log-phase cells grown at this temperature. On the other hand, transcript levels of *oppA*, *opuCA*, and *fri* in cells grown at 4°C in BHI were not affected by growth phase. Further, transcript levels for *opuCA* and *fri*, both of which are preceded by a σ^B-dependent promoter, were not lower in the Δ*sigB* null mutant. This observation was particularly striking for *opuCA*, which clearly shows σ^B-dependent transcription at 30 and 37°C (25, 34) and encodes a carnitine transporter that appears to contribute to cold growth in *L. monocytogenes* (2). Based on our promoter-mapping experiments, it appears that *opuCA* transcription originates predominantly from a σ^A-dependent promoter during growth at 4°C. Overall, it appears that the transcription of selected genes that encode proteins with roles in cold growth and that are partially σ^B dependent (e.g., *opuCA*, *fri*, and *ltrC*) is driven predominantly from σ^B-independent promoters in *L. monocytogenes* grown at 4°C. It is possible that activation of σ^B would contribute to enhanced transcription of these genes at 4°C if *L. monocytogenes* was exposed to other stress conditions reported to activate σ^B in *L. monocytogenes* grown at 30 or 37°C, e.g., nutrient limitation, acid stress, and osmotic stress (13, 15, 25, 35, 41). While contributions of σ^B to *L. monocytogenes* growth at 4°C may appear minor in cells grown in rich media, σ^B may contribute to the ability of *L. monocytogenes* to grow under refrigeration temperatures in at least some types of foods. Future experiments investigating the ability of an *L. monocytogenes* strain with a nonpolar *sigB* mutation to grow on different foods stored at refrigeration temperatures and investigating σ^B-dependent transcription in *L. monocytogenes* on foods stored at 4°C are needed to further clarify the contributions of σ^B to cold growth.

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